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Research Article

Human progenitor cells isolated from the developing cortex undergo decreased neurogenesis and eventual senescence following expansion in vitro

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ABSTRACT

Isolation of a true self-renewing stem cell from the human brain would be of great interest as a reliable source of neural tissue. Here, we report that human fetal cortical cells grown in epidermal growth factor expressed low levels of telomerase and telomeres in these cultures shortened over time leading to growth arrest after 30 weeks. Following leukemia inhibitory factor (LIF) supplementation, growth rates and telomerase expression increased. This was best demonstrated following cell cycle synchronization and staining for telomerase using immunocytochemistry. This increase in activity resulted in the maintenance of telomeres at approximately 7 kb for more than 60 weeks in vitro. However, all cultures displayed a lack of oligodendrotye production, decreases in neurogenesis over time and underwent replicative senescence associated with increased expression of p21 before 70 weeks in vitro. Thus, under our culture conditions, these cells are not stable, multipotent, telomerase expressing self-renewing stem cells. They may be more accurately described as human neural progenitor cells (hNPC) with limited lifespan and bi-potent potential (neurons/astrocytes). Interestingly, hNPC follow a course of proliferation, neuronal production and growth arrest similar to that seen during expansion and development of the human cortex, thus providing a possible model neural system. Furthermore, due to their high expansion potential and lack of tumorogenicity, these cells remain a unique and safe source of tissue for clinical transplantation.

Introduction

The expansion and differentiation of human stem cells in culture would provide a vital source of tissue for new types of cell therapy and disease modeling. However, human somatic cells in culture have a finite capacity for replication [1], and this senescence process is controlled by progressive telomere loss due to incomplete replication of the lagging DNA strand during each cell division [2]. The telomere hypothesis predicts that all somatic cells eventually senesce due to chromosomal end replication problems caused by telomeric erosion and/or uncapping (for review, see [3–5]). Mammalian germ line cells and most tumor cells express high levels of human telomerase reverse transcriptase (hTERT). This enzyme repairs the

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telomere units lost during every replicative cycle, thus allowing for unlimited growth [6].

Pluripotent human embryonic stem cells derived from the inner cell mass of the blastocyst have been shown to produce high levels of telomerase [7] and may indeed be immortal. Other somatic human stem cells, such as hematopoetic stem cells, express only very low levels of telomerase that is insufficient to maintain telomere length indefinitely in vitro [8,9]. These hematopoetic cultures have been very difficult to expand in culture even though their capacity for continual self-renewal is extensive in vivo. Human neural dividing precursors have also been generated from postmortem fetal brain (mainly cortex) that proliferate in response to either epidermal growth factor (EGF) or fibroblast growth factor 2 (FGF-2) [10–15]. Although these cultures are often described as neural stem cells, based on classical descriptions of the developing human cortex, it is most likely that many cells isolated between 8 and 20 weeks of development that respond to EGF or FGF-2 are also radial glial cells [16]. These have recently been shown to divide asymmetrically to generate new neurons [17] and may eventually give rise to astrocytes at later stages of development [18,19]. We have previously shown that these cultures initially express hTERT at very low levels that decrease to undetectable amounts following passaging [20]. However, no study to date has examined the exact lifespan of these cultures or the role of telomerase and telomere erosion during these extended growth periods.

Leukemia inhibitory factor (LIF), a member of the interleukin-6 family of cytokines, plays an important role in the self-renewal of mouse embryonic stem cells, but interestingly not human embryonic stem cells (for review, see [21,22]). LIF also has significant effects on the developing nervous system [23,24]. Addition of LIF to human neural precursor cells grown as aggregates termed "neurospheres" in the mitogen FGF-2 can increase expansion rates and allow long-term growth, although no effect was found when LIF was added to the same cells grown in the mitogen, EGF [12]. We have recently shown that if cell-to-cell contact is maintained by a unique passaging method which does not involve dissociation or trypsin treatment, EGF can maintain human neurosphere cultures for up to 100 days of growth [25]. Addition of LIF could further increase growth rates, and this change was associated with upregulation of a specific set of genes as shown by micro-array experiments [26]. However, telomerase expression was difficult to detect using gene chip analysis.

Here, we tested the hypothesis that LIF and EGF drive a self-renewing stem cell expressing telomerase, capable of producing unlimited numbers of neurons, astrocytes and oligodendrocytes. Although we show that LIF increased both proliferation and telomerase expression to allow significant expansion of the cells, eventual senescence could not be avoided. Furthermore, there was an absence of oligodendrocyte production after a few passages and a significant decrease in neurogenesis at later stages of expansion. Thus, the majority of cells grown under the current culture conditions appear to follow normal cortical developmental patterns and are most accurately defined as neural progenitors with limited expansion potential rather than true, self-renewing multipotent stem cells.

Results

LIF induces proliferation of telomerase-positive cells and attenuates telomere loss in hNPC cultures

We have previously shown that LIF could increase overall growth rates in three independent human neurosphere lines grown using a unique chopping method where cell/cell contact is constantly maintained [26]. In the current study, neurospheres were generated from an additional eight human fetal cortical samples (A–H) at 8 to 13 weeks of gestation (Table 1). These cultures were grown in serum-free, defined media supplemented with EGF, FGF-2 and heparin for the first 4 weeks and then switched to media supplemented with EGF alone and passaged by chopping without enzyme digestion or cell dissociation. After a total of 15 weeks in culture, a portion of each line was switched to media supplemented with EGF and LIF and designated "long-term LIF." The remaining portion was maintained in media supplemented with EGF alone and designated "never in LIF." At 30 weeks of total expansion, changes in neurosphere volume over a 13-day period were measured as an index of growth rate (Fig. 1a). The average sphere volume increase for long-term LIF-treated lines was threefold higher than sister cultures never in LIF (1180% ± 178 vs. 390% ± 130) confirming our previous results. All of the cultures maintained solely in EGF grew very slowly at this stage and eventually senesced in the absence of LIF [26]. See Supplementary Fig. 1 for individual growth curves for hNPC lines A–H.

One important determinant for cellular senescence in somatic cells is telomere length. Most human cells lack telomerase activity, and therefore, telomeres shorten at each cell cycle until reaching a critical length that activates cell arrest pathways. We have previously shown that telomerase activity was present in early passage hNPC cultured in EGF and FGF-2 but eventually declined to undetectable levels [20]. In order to determine if the extended viability of long-term LIF-treated cultures when compared to cultures maintained solely in EGF was associated with an increase in telomerase

Table 1 – Source and gestational age for hNPC lines

Designation	hNPC line	Source of tissue	Gestational age (weeks)
A	K031	University College, London	8
B	K054	University College, London	13
C	K052	University College, London	11
D	M031	University of Washington	13
E	K057	University College, London	10
F	M038	University of Washington	13
G	K066	University College, London	9
H	M006	University of Washington	12

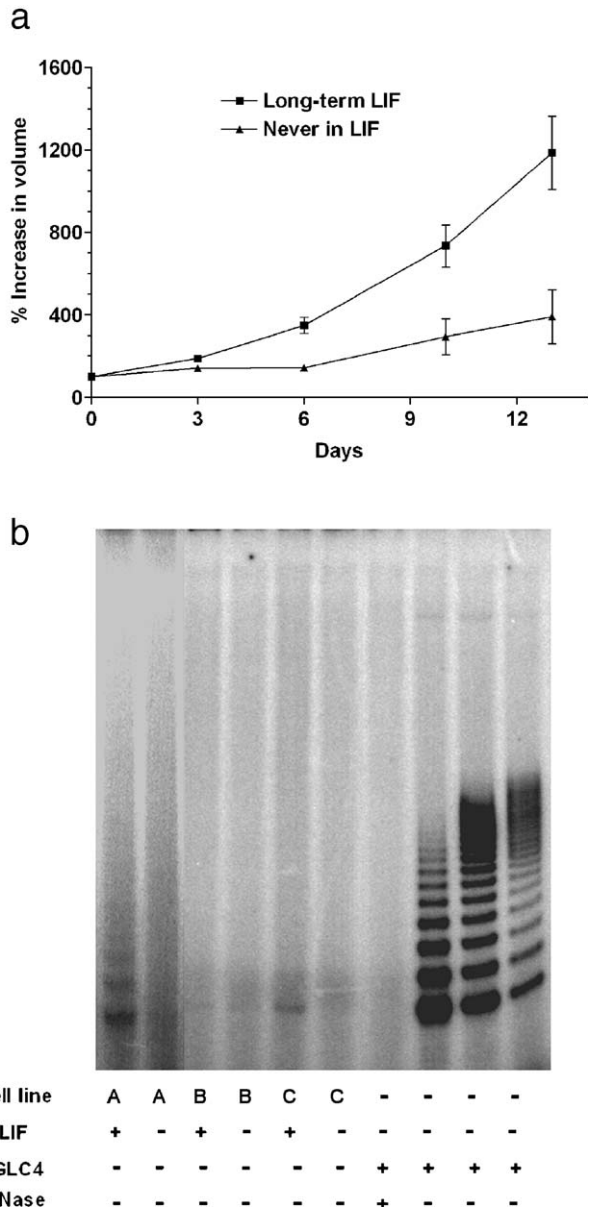


Fig. 1 – LIF-dependent increases in growth rate and telomerase activity in hNPC. Eight independent hNPC lines were grown initially in media supplemented solely with EGF. After 15 weeks, the lines were switched to media supplemented with EGF and LIF for 15 weeks (long-term LIF) or maintained in EGF alone (never in LIF). (a) Growth rates were determined by measuring changes in individual neurosphere volume over a 13-day period. (b) Telomerase activity was determined in cell extracts from three long-term LIF cultures (A, B, C) and in sister cultures grown in EGF only (never in LIF) using TRAP assay. GLC4 cells (1000, 200 and 40 ng per assay) or 200 ng GLC4 cell extract treated with RNAse served as controls.

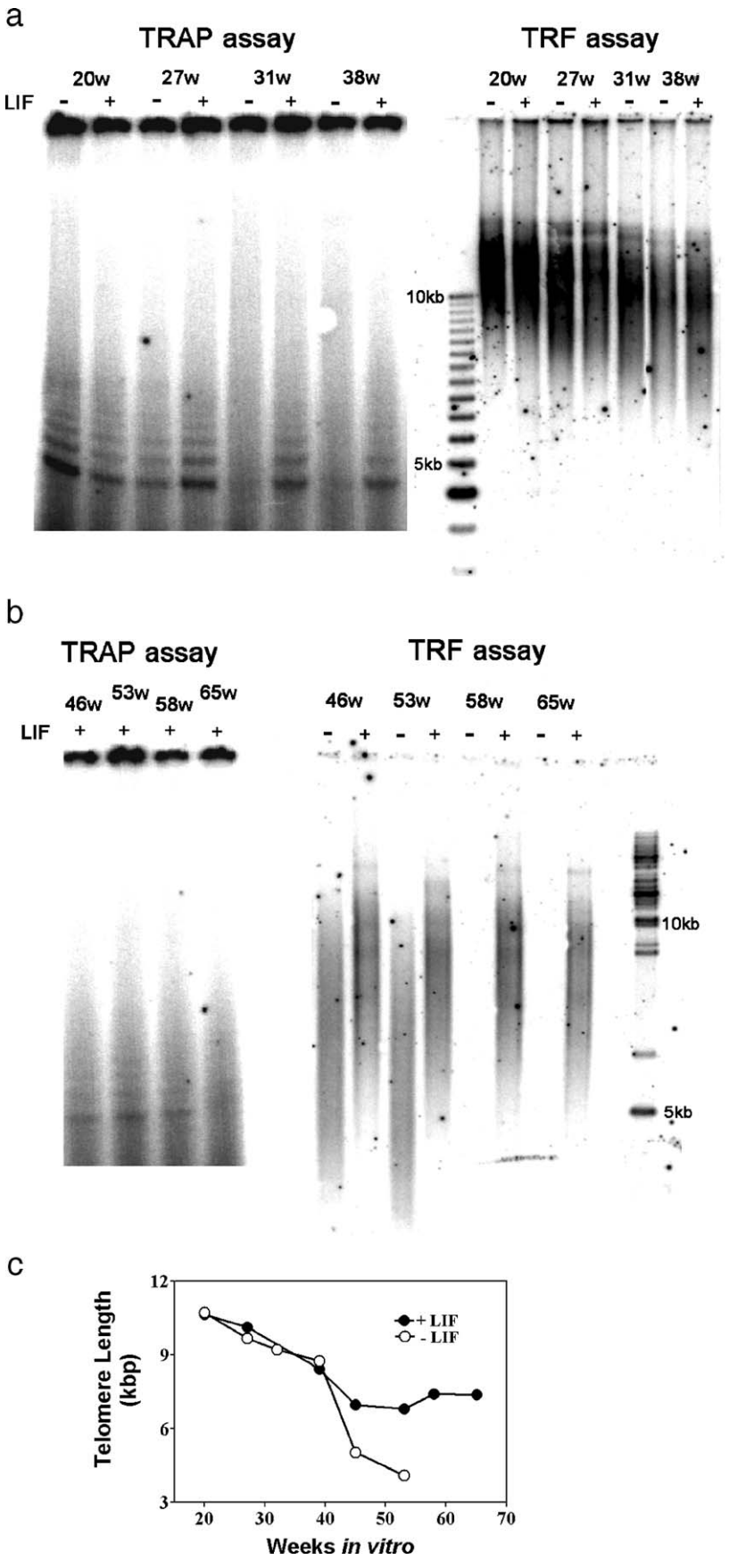
expression, we examined telomerase activity and protein levels in three hNPC lines (A, B and C). Cell lysates were assayed using a telomeric repeat amplification protocol (TRAP) to detect telomerase activity. Although robust activity was seen in the human lung cancer line, GLC4, TRAP activity was below the limits of detection for all of the cultures maintained solely in EGF (Fig 1b). In the corresponding long-term LIF-treated sister cultures, line A showed moderate telomerase activity, line B showed almost no activity, and line C displayed weak activity (Fig. 1b). Thus, detectable levels of telomerase activity were observed in some but not all long-term LIF-treated hNPC lines using this protocol and assay technique showing that there is some heterogeneity between individual fetal cortical samples. This variation may reflect similar heterogeneity between individuals with regards to telomerase activity.

We next asked how telomerase function was related to telomere length over time in the absence and presence of LIF. Line A (which showed the greatest telomerase expression) was expanded in media supplemented solely with EGF and cryopreserved after 14, 21, 25 and 32 weeks in culture. All of these frozen cells were thawed simultaneously and grown for an additional 6 weeks for a total culture time of 20, 27, 31 and 38 weeks in either EGF alone or EGF + LIF (Fig. 2a). The cultures were then assayed for telomerase activity using TRAP and also for average telomere length using the TRF assay. Although there was some telomerase expression at early passage number, no telomerase activity was detectable in cultures grown in EGF alone by 31 weeks. However, in the same cultures treated with LIF for 6 weeks, telomerase activity was maintained throughout the time course (Fig 2a). Interestingly, even if LIF was added to cultures grown in EGF alone for 31 weeks that lacked telomerase expression, some activity was found 6 weeks later. These data suggest that LIF may actually induce telomerase expression rather than just promote survival of a telomerase-positive cell. Nevertheless, this exposure to LIF and increased telomerase activity was not sufficient to repair to prevent telomere shortening (Figs. 2a, c).

Following these experiments, the 38-week cultures were maintained in culture until senescent and were assayed at 4 late-stage time points for telomerase activity or telomere length. After 40 weeks in vitro, the line supplemented with solely with EGF entered replicative senescence as characterized loss of proliferative potential (Supplementary Fig. 2) and significantly shortened telomeres but remained viable for an additional 10 weeks with some cell division during which time the remaining cells showed further rapid telomere shortening (Figs. 2b, c). In contrast, the LIF-treated culture continued to proliferate at 65 weeks (Supplementary Fig. 2), displayed telomerase activity until the last time point (65 weeks) in the study (Fig. 2b) and stopped growing at 75 weeks (data not shown). Interestingly, telomere length continued to shorten until reaching ~8 kb at which time there was no further erosion until the line became senescent.

Telomerase levels within individual cells are increased in synchronized hNPC

We next used immunocytochemical staining with a specific antibody to hTERT (the catalytic subunit of telomerase) to identify specific telomerase-expressing cells. Because few cells remained from the original lines (A, B and C), three new neurosphere lines (D, E and F) were generated (see Table 1). These lines were grown for 15 weeks in media supplemented solely with EGF, and then a portion of those cultures were supplemented with EGF and LIF (long-term LIF). The remaining


a
TRAP assay
TRF assay
20w 27w 31w 38w
LIF - + - + - + - +
20w 27w 31w 38w
- + - + - - - +
10kb
5kb
b
TRAP assay
TRF assay
46w 53w 58w 65w
LIF + + + +
46w 53w 58w 65w
- + - + - + - +
10kb
5kb
c
Telomere Length (kbp)
12
9
6
3
20 30 40 50 60 70
Weeks in vitro
+ LIF
- LIF

portion was maintained with EGF only (never in LIF). After a total of 30 weeks, a fraction of these cultures were dissociated, plated to coverslips and immunostained for hTERT. Strong staining was detected in the nuclei of the K562 human leukemia cell line used as a positive control (Fig. 3a). In all cultures grown solely in EGF, hTERT was only rarely detected (Fig. 3b). In the long-term LIF-treated cultures, hTERT protein was expressed at high amounts in the nuclei of many cells of line D (Fig. 3c). However, far fewer cells were positive for hTERT in lines E and F grown long-term in LIF (see Fig. 3f). These data support the individual variations observed in telomerase activity in the initial three lines studied (A, B and C) using the TRAP assay.

In a recent report, it was shown that although hTERT is typically indiscernible in normal human fibroblast lines [27], its expression is entrained to the cell cycle and can be detected when cells were in S-phase. We hypothesized that the variations in activity and protein we observed may be explained by the proportion of hNPC in S-phase at the time of sample preparation. Neurospheres from 3 hNPC lines (D, E and F) grown for 30 weeks long-term in LIF or solely in EGF (Never in LIF) were synchronized by the addition of the S-phase blocker, aphidicolin, at 5 μg/ml for 24 h, released from block for 24 h and cell cycle distribution analyzed by flow cytometry. Representative histograms from cell cycle analysis (line D, never in LIF) for unsynchronized hNPC and aphidicolin block/release are shown in Figs. 3d and e. In unsynchronized cells, 8.3% of total cells were in S-phase compared to 38.9% after synchronization treatment. Another S-phase block (5 mM thymidine) gave similar results (data not shown). When synchronized and unsynchronized cells from these 3 lines (E, F, G) were fixed and immunostained for hTERT, there was a striking increase in the percent of hTERT labeled cells in unsynchronized cultures of line D grown in EGF solely, but not lines E and F (Fig. 3f). However, all long-term LIF-treated cultures showed significant increases in hTERT expression upon synchronization with the percentage of hTERT immunopositive cells increasing in line D from 65.0% ± 4.3 to 81.3% ± 2.4 ($P < 0.001$), line E from 2.5% ± 1.0 to 17.3% ± 1.5 ($P < 0.01$) and line F from 2.4% ± 0.5 to 8.5% ± 2.9 ($P < 0.05$) (Fig. 3f). These data establish that there is dynamic regulation of telomerase expression within dividing hNPC through the cell cycle. Furthermore, it demonstrates that even in lines with low basal levels of hTERT, LIF can cause significant increases in telomerase protein levels. As cells in S-phase express higher levels of hTERT, it is possible that by simply increasing proliferation, LIF-treated cultures contain a high proportion of cells expressing telomerase.

LIF dynamically modulates telomere length in hNPC cultures

Having established that LIF could increase telomerase in dividing hNPC following synchronization, we next compared the telomere length in lines D, E and F grown for a total of 30 weeks in the presence or absence of LIF as described above. Interestingly, line D which had the highest telomerase expression also had the longest telomeres (Fig. 4a). This line also had the fastest population doubling times (Table 2). For each line, the long-term LIF-treated cultures had an average telomere length at least 1 kb longer than that of the corresponding sister culture never in LIF (Fig. 4a) confirming our previous data in Line A (see Fig. 2).

We next investigated the dynamic correlation between LIF-mediated proliferation and attenuation of telomere length. Line D was grown for 30 weeks solely in EGF (never in LIF) or in long-term LIF. Proliferative rates using BrdU incorporation confirmed that cultures in LIF were dividing more frequently (Fig. 4b: 26.7% ± 1.16 vs. 16.5% ± 0.05, $P < 0.0001$). When LIF was withdrawn for 6 weeks from long-term LIF-treated cells in culture for a total of 30 weeks, the growth rate decreased to levels similar to the sister cultures never in LIF (14.4% ± 1.04, $P < 0.005$ compared to long-term LIF). Likewise, when LIF was added for 3 weeks to naïve cultures grown in EGF alone for 30 weeks, BrdU incorporation increased to a level (22.4% ± 1.03, $P < 0.0001$ compared to culture never in LIF) approaching that of long-term LIF treatment (Fig. 4b). These results suggest that LIF had a potent effect on the proliferation in these cultures even after long growth in EGF alone. When telomere length was analyzed for these treatments, the withdrawal of LIF from long-term cultures increased telomere erosion and the addition of LIF to naïve sister cultures attenuated the erosion (Fig. 4c, Supplementary Fig. 3). The effect of LIF on telomere length was dynamic, and these results mirror the changes in proliferation described in Fig. 4b. Similar correlations between the addition of LIF and telomere erosion were also observed for lines E and F (Supplementary Fig. 3, Supplementary Table 1). This data shows that LIF can induce proliferation of hNPC, leading to activation of telomerase and concomitant repair of the telomeres.

LIF does not prevent the eventual senescence of hNPC cultures

Do these telomerase-positive cells grown in LIF avoid cellular senescence? To address this question, we monitored the growth rates for six long-term LIF-treated lines (A–F) over time. Eventually, every line senesced at approximately 50 to 75 weeks of growth. To examine these observations in

Fig. 2 – LIF maintains telomere length in hNPC over an extended culture time. (a) Telomerase activity (TRAP assay) and telomere length (TRF assay) were assessed in cultures expanded for up to 38 weeks (w). Line A was grown in media supplemented solely with EGF and cryopreserved after 14, 21, 25 and 32 weeks in culture. Frozen cells were thawed simultaneously and grown for an additional 6 weeks for a total culture time of 20, 27, 31 and 38 weeks, respectively in the original media supplemented with EGF alone (–) or in media containing EGF and LIF (+) and TRAP and TRF assays were performed. (b) The oldest culture (38 weeks) was maintained for an additional 27 weeks, and TRAP and TRF assays were done at 46, 53, 58 and 65 weeks on this line. Note that cultures supplemented solely with EGF grew very slowly after 40 weeks, and there was insufficient material for testing at the later time points. (c) Plot of average telomere length for line A comparing long-term LIF addition (+LIF) to sister cultures grown solely in EGF (–LIF).

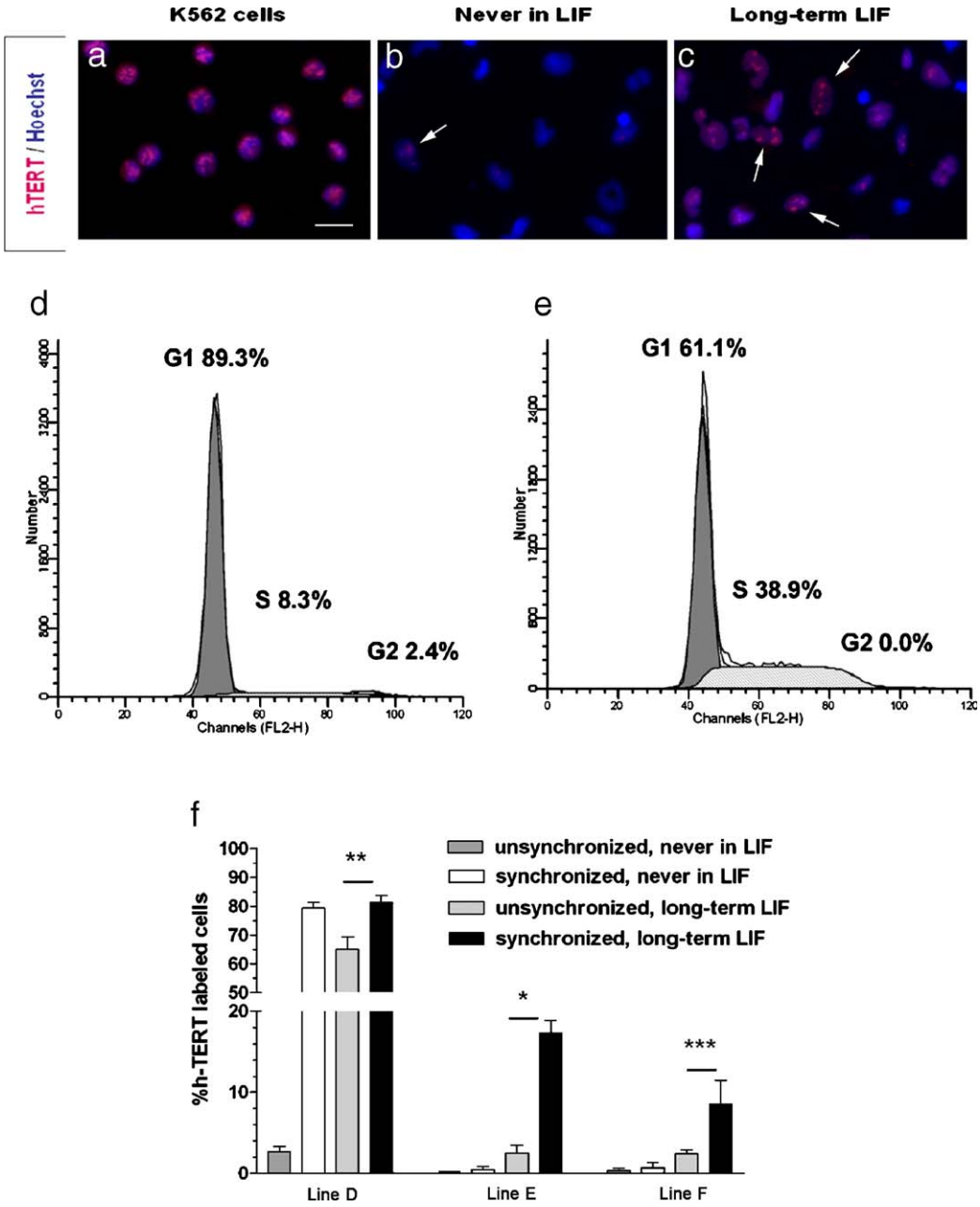


Fig. 3 – Increased telomerase expression in synchronized hNPC. Neurospheres were dissociated into single cell suspension, plated for 1 h prior to fixation and immunostained with anti-hTERT (red), and nuclei were counter-stained with Hoechst dye (blue). (a) K562 cells served as positive control. (b) hNPC from line D grown solely in EGF (never in LIF). (c) Line D grown long-term in LIF. Arrows designate representative immunopositive nuclei. Scale bar = 10 μm. Long-term LIF hNPC lines (D, E, F) and sister cultures never in LIF were synchronized by addition of aphidicolin (5 μg/ml) for 24 h, released from block for 24 h, and DNA content analyzed by flow cytometry. Histograms of cell cycle analysis of (d) unsynchronized line D (never in LIF) and (e) aphidicolin-synchronized line D (never in LIF) 24 h after release from block. (f) At the end of the release period, cultures were dissociated into single cell suspension, plated for 1 h prior to fixation and the percentage of hTERT-immunopositive cells was determined. All data are expressed as means ± SEM. $^{*}P < 0.0001$, $^{**}P < 0.01$, $^{***}P < 0.05$ from unsynchronized control.

greater detail, one additional line (G) was isolated and assessed from the start of culturing. LIF was added following 15 weeks growth in media supplemented with EGF alone. Growth rates were assessed by measuring increases in sphere volume and BrdU incorporation following a 14-h pulse, and telomere length measurement by TRF assay was performed at 30, 40, 50, 60 and 70 weeks of culture. By 50 weeks, there was a steady decline in proliferation as measured by both growth parameters, and by 70 weeks, there was very little further growth of this line (Figs. 5a and b). This line also showed a steady decrease in telomere length over time (Figs. 5c and d). However, there was no dramatic shortening at 40 weeks to lengths seen for EGF alone (see Fig. 2) but rather a gradual reduction towards the

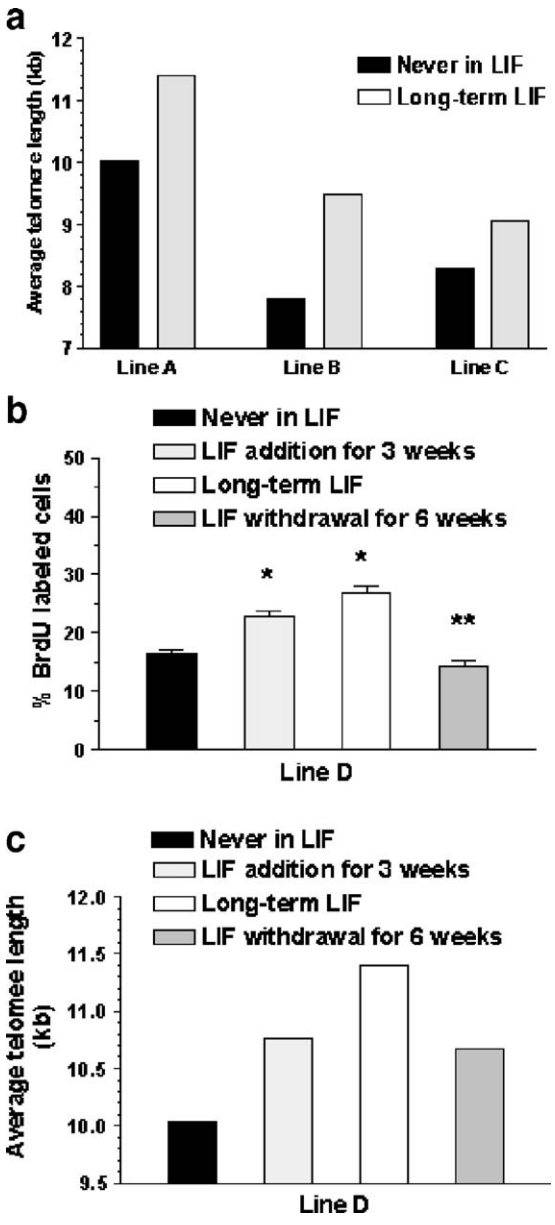


Fig. 4 – LIF dynamically modulates telomere length. (a) Average telomere length was determined by TRF analysis in cell extracts from three long-term LIF cultures after 30 weeks in vitro (D, E, F) and in sister cultures never in LIF. (b) Proliferation rates for line D were assessed in cultures grown solely in EGF (never in LIF), in cultures with LIF added for 3 weeks following supplementation solely in EGF for 30 weeks in cultures, in sister cultures grown long-term in LIF and cultures withdrawn from LIF for 6 weeks after long-term LIF for 30 weeks in vitro. Cultures were pulsed with 0.2 μM BrdU for 14 h prior to harvest and the percentage of BrdU-immunopositive cells was determined. All data are expressed as means ± SEM (*$P<0.0001$ from culture never in LIF, **$P<0.003$ from long-term LIF culture). (c) Average telomere lengths for those treatments were determined by TRF assay.

end of its growth phase to a length of approximately 8 kb (Figs. 5c and d) in agreement with the other long-term LIF-treated line A (see Fig. 2).

We next asked whether p21, a gene involved with cell cycle arrest, was up regulated when the cells became senescent. Protein lysates were prepared from lines D, E and F grown solely in EGF and never in LIF (35 weeks in vitro), from long-term LIF-treated cultures at early passage (25 total weeks in vitro) and at late passage (50 total weeks in vitro) and immunoblotted for p21. Interestingly, p21 expression was dramatically increased in lines never grown in LIF when they reached senescence at approximately 35 weeks (Fig. 5e). While early passage LIF-treated cultures did not express p21, it was seen at high levels in lines D and E at late passage (Fig. 5e). Interestingly, Line F which grew slowly was already showing low but significant p21 expression when grown with LIF at 25 weeks which remained stable at later passages.

To confirm that the decline in growth rate was due to cell cycle arrest, population doubling times (PD) were determined for lines E, F and G grown for 30 weeks in media supplemented with EGF alone or sister cultures grown long-term in LIF. Viability in all cultures was greater than 95% as estimated by trypan blue exclusion. Although there was some variation between the different cell lines, PD rates were 70 to 86% longer in each of the naïve cultures compared to the sister cultures grown long-term in LIF for the same length of time (Table 2). In addition, when flow cytometry was used to examine DNA content as an index of cell cycle distribution for line D at early (25 weeks in vitro) and late passage (50 weeks in vitro), there was a significant decrease in the percentage of cells in S-phase (Table 3) in the late passage culture that corresponded with the increase in p21 expression. Together, these results show that hNPC expressing low levels of telomerase are not immortal and undergo normal senescence as they reach a critical stage in culture.

Long-term LIF-treated cultures showed reduced neurogenesis at late passages

Human neurospheres undergo neurogenesis when plated onto attachment substrates in the absence of mitogens [28]. To examine the effect of LIF on neurogenesis, individual neurospheres from the 8 lines described in Table 1 were plated on a laminin/poly-L-lysine-coated surface, cultured

Table 2 – Population doubling times for three hNPC linesafter 30 weeks in culture

Cell line	Culture conditions	PD (days)	Fold increase
D	Long-term LIF	4.28 ± 0.26	–
D	Never in LIF	7.24 ± 0.69*	1. 69
E	Long-term LIF	5.28 ± 0.49	–
E	Never in LIF	8.24 ± 0.53**	1.53
F	Long-term LIF	6.14 ± 0.36	–
F	Never in LIF	11.45 ± 0.51*	1.86

Individual neurospheres (n = 10) from 30-week long-term LIF-treated lines and sister cultures supplemented solely with EGF (never in LIF) were selected, diameters measured at 6 and 10 days, total number of cells per sphere estimated, and mean generation times (PD) determined. Data are expressed as means ± SEM; $P < 0.001$, **$P < 0.003$ compared to long-term LIF sister culture.

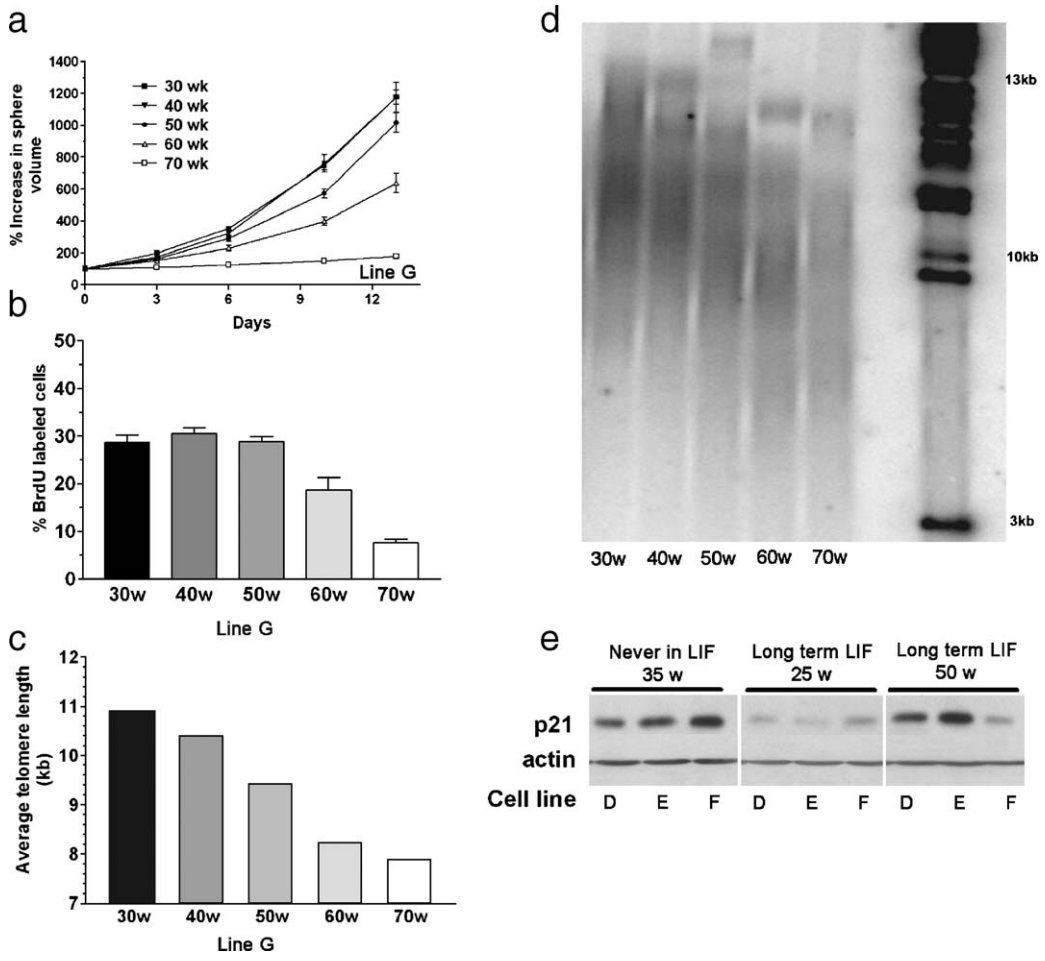


Fig. 5 – Addition of LIF does not prevent eventual senescence of hNPC. A single hNPC line (G) was supplemented with LIF after 15 weeks in culture and growth rates were determined every 10 weeks by (a) measuring changes in individual neurosphere volumes over a 13-day period and (b) BrdU incorporation following a 14-h pulse. (c) Average telomere length was determined by TRF assay for line G at 30, 40, 50, 60 and 70 weeks in vitro. (d) Phospho-Imager radiogram of TRF assay. (e) A Western blot was prepared from 3 hNPC lines (D, E, F) at early passage LIF-treated (25 weeks in vitro), late passage LIF-treated (50 weeks in vitro), and senescent cultures never in LIF (35 weeks in vitro) and probed with antibodies toward p21 and actin.

in the absence of growth factors for 14 days, fixed and then immunostained for the neuronal marker, β-tubulin III. The number of neurons generated from LIF-treated cultures at 30 weeks of expansion was twofold higher than sister cultures never in LIF (Fig. 6a: 54.8% ± 3.3 vs. 28.8% ± 4.6, $P < 0.001$). We next established whether cultures grown long-term in LIF showed any changes in neurogenesis as they aged in culture. Individual neurospheres from line G grown long-term in LIF as described earlier were selected every 10 weeks, differentiated for 14 days and then immunostained for β-tubulin III (Figs. 6b and c). Although the number of neurons generated remained stable (approximately 50% of total cells) until at least 40 weeks (53.1% ± 1.9 β-tubulin III immunopositive cells), a progressive decrease in neurogenesis was detected at 50 weeks (31.6% ± 2.3) through 70 weeks (9.9% ± 1.1) (Fig. 6b). The other seven lines showed a similar loss of neurogenic potential (data not shown). Thus, as in human cortical development, hNPC show a gradual decrease in neurogenesis over time.

Discussion

Neural progenitors isolated from the human fetal brain and grown as neurospheres have a finite lifespan in vitro. In cultures maintained in EGF alone, telomeres shorten and the cells senesce within 40 weeks of culture time. LIF administration increases telomerase expression and maintains telomere length but does not prevent eventual senescence or the loss of neurogenic potential. In addition, this growth arrest is associated with increases in cell cycle times as well as proteins involved in replicative senescence such as p21. We propose that as grown under the current conditions, these cells are best described as human neural progenitor cells rather than self-renewing stem cells.

It is important to note that there are major differences between rodent and human cells with regard to regulation of telomere length and telomerase biology. Dividing somatic neural precursor cells derived from rodents have long

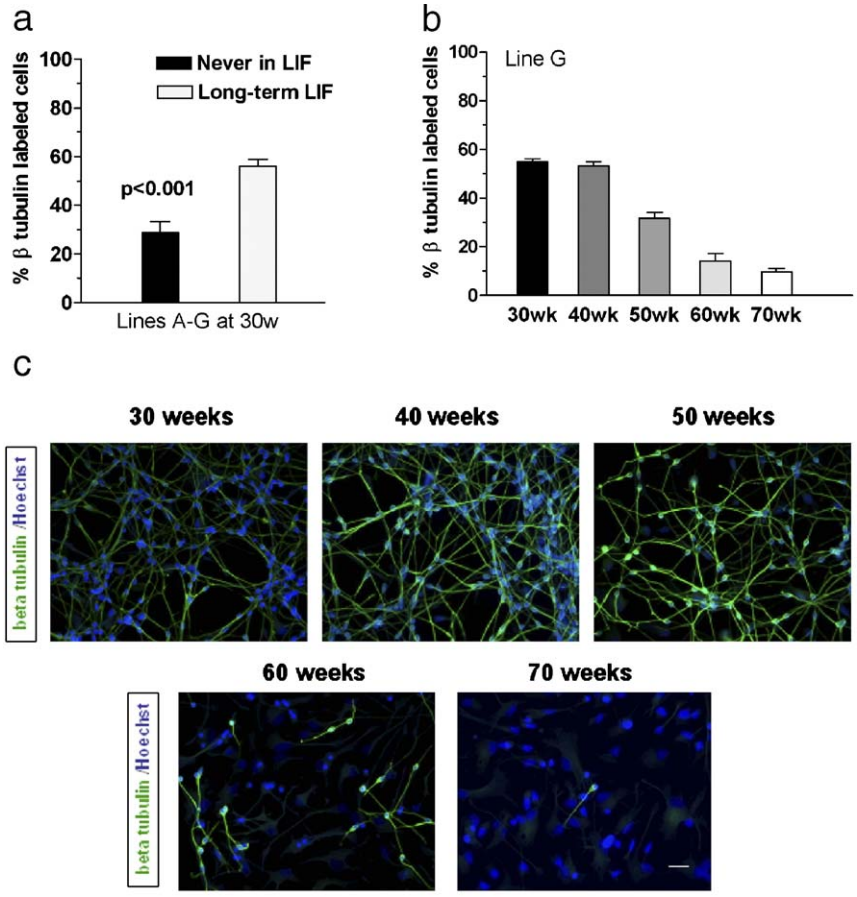


Fig. 6 – LIF-dependent increases in neurogenesis decline over time. Eight hNPC lines (A–H) were grown initially in media supplemented solely with EGF. After 15 weeks, a portion of each line was supplemented with EGF and LIF, and all lines were grown for an additional 15 weeks. (a) Neurospheres from these lines were differentiated in the absence of mitogens for 14 days and percentage of neurons determined by immunostaining for the neuronal marker β-tubulin III. (b) A single hNPC line (G) was supplemented with LIF after 15 weeks in culture, and every 10 weeks, the amount of neurogenesis was assessed after differentiation for 14 days by determining the percentage of β-tubulin III immunopositive cells. Data are expressed as means ± SEM. (c) Photomicrographs of the time course of differentiation of line G immunostained for β-tubulin III (green) and nuclei counter stained with Hoechst (blue). Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

telomeres and express high levels of telomerase [20], further upregulate telomerase in response to FGF-2 [29], and have been shown to have a limitless capacity for growth providing the culture conditions are optimal [30]. In contrast, dividing human cells express low levels of telomerase, have shorter telomeres and as such may be directly affected by telomere erosion [20,31]. Here, we show that in hNPC grown in EGF alone, there is a strong senescence pattern that occurs at around 40 weeks that is associated with lack of telomerase expression and reduced telomere length to ~ 4 kb. Under our culture conditions, no cells within the neurospheres were found to spontaneously immortalize and continue growing in the absence of LIF (based on more than 20 lines grown for more than a year in vitro). Thus, sporadic immortalization is likely to be a very rare event as described previously for other human cell types [32]. Our method of growing the cells does not involve weekly treatment with enzymes and dissociation to single cells or selection based on surface or other markers as used by many other studies [14,15]. However, while these approaches can enrich specific types of precursor cell from primary fetal tissue cultures, there are no reports that they can undergo continual self-renewal past a few passages without losing phenotypic potential.

In initial studies, we found that LIF increased cell proliferation rates of all hNPC lines and induced the expression of telomerase in some of these lines. However, the variation in overall telomerase activity and poor sensitivity of the TRAP assay led us to test other more sensitive methods of detecting this protein. Human fibroblasts are considered to lack hTERT expression; however, when synchronized, telomerase activity can be detected at S-phase in these cells [27]. Likewise, when hNPC were synchronized using the S-phase blocker, aphidicolin, the number of immunopositive cells dramatically increased in all long-term LIF cultures examined. Thus, there is a clear correlation between cell division and telomerase expression. It is possible that LIF acts by simply increasing proliferation of hNPC and that dividing hNPC express higher levels of telomerase allowing longer expansion in culture.

Table 3 – Cell cycle distribution for early and latepassage hNPC

Cell line	G1/G0	S	G2/M
Line D, early passage (25 weeks)	76.5 ± 0.2	18.1 ± 0.2	4.7 ± 0.9
Line D, late passage (50 weeks)	89.0 ± 0.4	8.6 ± 0.4*	2.4 ± 0.1

hNPC at 25 and 50 weeks in vitro were fixed, stained with propidium iodide and DNA content analyzed by flow cytometry. Assays were run in duplicate. Data are expressed as means ± SD.
*$P < 0.0001$ compared to early passage.

While this increase in telomerase expression could increase overall telomere length over short time periods, it was not sufficient to prevent eventual telomere erosion in the absence or presence of LIF following continual growth. Other short-term expansion studies on hematopoetic stem cells have also shown that various combinations of cytokines can increase telomerase levels in early passage cells, but in agreement with the current data, this change was not associated with telomere maintenance [8]. Why these modest telomerase increases are not sufficient to maintain telomeres is not presently known, but similar observations have also been noted for human keratinocytes [32].

Unlike blood cells, human neurospheres can be grown for long periods of time, and we were therefore able to show that LIF induced telomerase expression was able to maintain a consistent telomere length of ~ 7 kb for up to 70 weeks of growth. Furthermore, cultures with short telomeres "repaired" to some extent upon LIF administration although not back to the 12 kb seen in primary cultures. These dynamic changes in telomere length demonstrate that there is an active process of hTERT-dependent telomere maintenance in long-term LIF cultures. In human ovarian cancer cells, hTERT expression is induced by the Akt cascade [33]. LIF has been shown to activate Akt in cardiac myoblasts and fibroblasts [34,35]. One possible mechanism for LIF modulation of hTERT expression in hNPC may through a LIF-dependent activation of Akt. It has also been reported that telomerase can mediate cell survival [36], and this synergistic effect may also play a role in the prolonged viability of LIF-treated hNPC. These findings demonstrate that telomerase is present in hNPC and plays an important role in the longevity of these LIF-treated cultures.

Another explanation may be that the increase in telomerase activity is the result of increased proliferation and a bigger population of S-phase cells expressing telomerase rather than a direct consequence of LIF treatment. In an earlier study, we showed that the addition of LIF increased growth rates in hNPC [26], and, in the current study, the withdrawal of LIF from the long-term cultures as well as the addition of LIF to naïve lines had a significant effects upon growth rates (Fig. 4b) with concomitant effects on telomere length. The addition of LIF attenuated the rate of erosion seen in the naïve cells in contrast to the accelerated loss of telomeres when LIF was removed. Likewise, when population doubling times and telomere lengths were compared for lines D, E and F, the fastest growing line (D) had the longest telomeres whereas the slowest (line F) had the shortest. These observations suggest that the effects of LIF on telomere length may be entrained to the growth rate. In contrast to the typical loss of telomere units that occurs at each cell division, the increased amount of telomerase expressed at S-phase seen only when LIF is present may slow that progressive loss. Therefore, whether by direct activation of a signaling pathway or by an indirect action through expanding S-phase, exposure to LIF increases telomerase activity within these cultures. However, when growth rates from individual lines (Supplementary Fig. 1) were compared with the corresponding TRAP assay results for those lines, Line B demonstrated the fastest growth rate but had undetectable telomerase activity, whereas the slower growing lines A and C had measurable amounts of activity. Also, of interest is the fact that although lines D and F were generated from tissue of identical gestational age, there was more than 2 kb difference in the average telomere length after identical time in vitro and culture conditions. These findings suggest that the inherent variation in individuals is also important in telomere regulation as shown previously for other tissues [37,38].

Nevertheless, hTERT expression was insufficient to maintain telomere length and even long-term LIF-treated lines eventually senesced. Downstream, cellular senescence is controlled by two tumor suppressor pathways, p53 and Rb. The p53 effector, p21, acts as a potent cyclin-dependent kinase inhibitor and, in hNPC, p21 was shown to be increased in a manner consistent with the canonical p53 senescence pathway. Increases in the population doubling times and decreases in the percentage of cells in S-phase in these samples indicate a cellular senescence program mediated by p53. Herbig and colleagues have shown that sites of DNA damage localized to the telomeres of senescent human fibroblasts correlated with ATM, p53 and p21, but not p16 [39]. In addition, the expression of telomerase itself has been also shown to be repressed by a p53-dependent mechanism in mediated through p21 acting upstream from E2F [40]. Both of these events may act in concert to promote senescence. Interestingly, even though line A expressed the highest levels of hTERT, it still followed a similar time course of growth arrest demonstrating that the increases in hTERT were not indicative of a transformation event. Indeed, all of the 150 hNPC lines isolated using our methodology have exhibited similar patterns of growth and senescence. Furthermore, even though hundreds of rodents have received hNPC transplants, no tumors have been observed to date testifying to the safety of these cells following grafting.

Would it be possible to avoid this inherent senescence pattern and allow continual growth of these cells? hTERT overexpression at high levels has been shown to be sufficient for immortalization for several cell types. For example, Roy et. al used retroviral hTERT overexpression to immortalize hNPC from human fetal spinal cord which were shown to maintain long-term growth for over 120 population doublings, retain a normal karyotype and survive transplantation [41]. This work is a very interesting approach and may be particularly appropriate for expanding regions of the developing human brain that are difficult to grow such as the spinal cord. In another study, very high hTERT levels were maintained for 4 years through a v-myc-mediated perpetuation [42]. Initially, it appeared that this way of immortalizing any primary cells occurred with the maintenance of normal phenotype [43–45].

However, it has also been reported that an hTERT transduced hNPC line gained an abnormal karyotype and formed neuroblastoma-like tumors after transplantation [46]. Furthermore, recent data indicate that other hTERT immortalized somatic cell lines show changes in cell cycle regulatory genes and karyotypes after prolonged culturing [47–50]. Using LIF treatment of hNPC to activate telomerase and thereby allow extended proliferative capacity may provide an alternative means for massive expansion without the risk associated with changing the genome by introducing the hTERT gene or other immortalizing agents.

There is currently some debate over the terminology of stem cells, progenitors and precursors. Our definition is in agreement with other recent reports [51–53] and requires that a neural stem cell must self-renew for long periods and retain the potential to generate neurons, astrocytes and oligodendrocytes. In contrast, a neural progenitor cell does not necessarily self-renew, often produces only limited neural phenotypes (uni-potential or bi-potential) and has limited expansion. Finally, a precursor cell is simply capable of proliferation. Previous reports on human neural precursor cells suggest that some of the requirements for stem cell classification can be met. For example, Uchida et al. demonstrated that CD133 expression defined a cell within the primary human fetal cortex that was clonogenic and could self-renew, although multipotency was not confirmed [14]. Keyong et al. isolated a nestin/musashi positive cell from the human fetal cortex that was also clonogenic and could produce astrocytes, neurons and rare oligodendrocytes for up to 6 months in culture [15]. It is possible that these cell divisions represent the normal radial glial progenitor cell expansion needed to produce the enormous number of cells required to create the cortex during the 9 to 12 month period necessary for human brain development. Furthermore, the lack of oligodendrocyte production following passaging in all previous published reports on human neural precursor cells suggests that cell division of a truly multipotent cell may be a rare event in vitro. Instead, most of these studies and the current paper are likely to be propagating neural progenitor cells, some with a radial glia phenotype. However, using these progenitors, we have been able to generate and bank over 100 billion cells. Providing they are used between passages 10 and 40, there is a very constant pattern of cell differentiation into neurons and astrocytes (Fig. 6). Thus, these cultures remain of great interest for use in human cell therapy applications where risk of tumor formation needs to be minimized [54].

Methods

Tissue collection

Human embryonic tissue (between 8 and 13 weeks post conception) was provided by Dr. Eric Jauniaux, Department of Obstetrics and Gynaecology, University College, London or from the Birth Defects Laboratory at the University of Washington. Full ethical approval had been granted by the Local Research Ethics Committee, University College Hospital, London, and the Human Subjects Committee at the University of Wisconsin. The methods of collection conform with the arrangements recommended by the Polkinghorne Committee and NIH for the collection of such tissues and to the guidelines set out by the United Kingdom Department of Health and the University of Wisconsin.

Cell culture

Eight hNPC lines (A–H) were isolated from human fetal cortex 6–13 weeks post conception and grown as free-floating neurospheres as previously detailed [26,55]. Freshly dissected tissue was sectioned into small cubes (1 mm^2) and seeded into T75 flasks at a density of 200,000 cells/ml of serum-free medium consisting of 70% Dulbecco's modified Eagle medium, 30% Hams F12, 1% penicillin/streptomycin/amphotericin B (basal media) supplemented with 2% B27 (v/v) (Life Technologies, Rockville, MD), 20 ng/ml epidermal growth factor (EGF, Sigma, St. Louis, MO), 20 ng/ml basic fibroblast growth factor (FGF-2, R&D Systems, Minneapolis, MN). Heparin (5 μg/ml) was added to stabilize the FGF-2 and increase initial growth rates [56]. All cultures were maintained in a humidified incubator (37 °C, 5% CO_2 in air), and half the growth medium was replenished every 3–4 days. Neurospheres were passaged every 10 to 14 days by sectioning of neurospheres into 200-μm sections that were seeded into fresh growth medium at a density equivalent to 200,000 cells/ml. At 2 weeks after the first passage, cells were switched to basal media containing 1% N2 (v/v) (Life Technologies, Rockville, MD) and 20 ng/ml EGF. At 15 weeks of growth, 10 ng/ml LIF (Chemicon, Temecula, CA) was added to the cultures. Typically, assay samples were collected at the midpoint between passages. In cases where LIF was withdrawn, neurospheres were collected, medium removed, and neurospheres washed three times with 1% N2 supplemented with 20 ng/ml EGF, and cultured into the same media. For differentiation studies, whole spheres were plated directly in 2% B27 in basal medium onto 0.01% poly-L-lysine and 10 μg/ml laminin-coated glass coverslips in 24-well plates. After 14 days, the spheres were fixed with 4% paraformaldehyde for 20 min, washed with PBS and immunostained for β-tubulin III.

Growth studies

Individual spheres (approximately 0.30 mm in diameter) for each treatment were transferred to a single well of a 96-well plate (n = 10) containing 200 μl of growth medium supplemented with the appropriate growth factor combination. Diameter measurements were taken every 3 to 4 days using a lens-mounted micrometer. The volume of each sphere was calculated as an index of cell number as previously described in detail [25]. For 5-bromo-2′-deoxyuridine (BrdU) incorporation assays, neurospheres were pulsed with 0.2 μM BrdU (Sigma, ST. Louis, MO) for 14 h before dissociation and plated to coverslips coated with 0.01% poly-L-lysine and 10 μg/ml laminin for 60 min before fixation as described below. Following fixation, cells were stained with 1:300 anti-BrdU (Roche, Indianapolis, IN) according to manufacturer's instructions. All data were expressed as means ± SEM and were analyzed using one-way ANOVA with Newman–Keuls post hoc test.

Cell counts

Cell counts were performed using a Nikon fluorescence microscope (40× objective) and Metamorph Imaging software (Universal Imaging Corporation, Downington, PA). Quantification of cells was based on counting the number of Hoechst-stained nuclei and the specified immunomarkers in at least 5 independent fields containing at least 60 cells per field (total area >25 mm^2) from a minimum of 3 coverslips. In the case of the differentiated neurospheres, at least 5 random fields were analyzed from the monolayer surrounding the plated sphere.

Immunocytochemistry

For studies examining proliferating hNPC, neurospheres were dissociated into a single cell suspension with Accutase (Innovative Cell Technologies, San Diego, CA) for 10 min at 37 °C and 30,000 cells were plated onto 0.01% poly-L-lysine and 10 μg/ml laminin-coated glass coverslips in 24-well plates and incubated for 60 min at 37 °C. The plating media consisted of 2% B27 in basal media. Cells were fixed with 4% paraformaldehyde for 20 min or ice-cold methanol for 5 min and washed with phosphate-buffered saline (PBS). Fixed cell cultures were blocked with 5% normal goat serum and 0.2% Triton x-100 in PBS and processed for immunocytochemistry with primary antibody to β-tubulin III (monoclonal, 1:5,000, Sigma, St. Louis, MO) or hTERT, the catalytic unit of telomerase (monoclonal, 1:500, Novocastra, UK). Following rinsing with PBS, the cells were incubated for 30 min with secondary antibodies conjugated to fluorescein or Cy3 (Jackson Immunoresearch Laboratories, West Grove, PA). Hoechst 33258 (1:10,000 in PBS) was added for 5 min after completion of the secondary antibody incubation as a nuclear stain. All cell count data were expressed as means ± SEM and analyzed using one-way ANOVA with Newman–Keuls post hoc test.

Telomeric repeat amplification protocol (TRAP) for hTERT activity

Neurospheres were rinsed in PBS, dissociated into a single cell suspension, and cell number was determined. Cells were suspended in CHAPS lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol) using 50 μl of lysis buffer per 10^6 cells and placed on ice for 30 min. Samples were centrifuged at 12,000×*g* for 30 min at 4 °C. Protein concentrations were determined using Biorad protein reagent (Biorad, Richmond VA), and TRAP assays were performed as described previously [57]. A aliquot of each protein extract (5 μg) was incubated with 100 ng of the TS oligo (5′-AATCCGTCGAGCAGAGTT-3′), buffer, 50 μM dNTPs, 1 U Taq polymerase and 0.4 μl of [α-^{32}P]dCTP (10 μCi/μl, 3000 Ci/mmol) for 15 min at room temperature. The tubes were transferred to a thermal cycler and heated for 10 min at 94 °C before adding 100 ng of the CX primer (5′-CCC TTACCCTTACCCTTACCCTAA-3′). After 27 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, half of the reaction was analyzed by electrophoresis in 0.6× Tris–boric acid–EDTA buffer (TBE) on 10% polyacrylamide non-denaturing gels. For quantification, gels were run in duplicate and exposed to a Phospho-Imager plate (Molecular Dynamics, Boston, MA). A human lung cancer line, GLC4, was used as a positive control.

Terminal restriction length fragment assay (TRF)

Neurospheres were washed with PBS, dissociated into a single cell suspension, cell number determined, genomic DNA extracted and average telomere length was determined by TRF assay [57]. Cells were resuspended in 100 μl of 1× Tris–EDTA (TE), added to 1 ml of DNA extraction buffer (10 mM Tris, 25 mM EDTA, pH 8, 0.5% SDS, 0.1 mg/ml Proteinase K) and digested at 50 °C for 16 h. After phenol extraction and ethanol precipitation, DNA was dissolved in 1× TE and heated at 50–55 °C for 1 h. An aliquot of DNA (3–5 μg) was digested with HinF1/RsaI (2 units of each per μg DNA) and DNase-free RNase at 37 °C for 16 h. Aliquots of digested DNA (1–2 μg) were separated on 0.5% agarose gels in 1× TBE. The gels were dried for 30 min at 60 °C, denatured in 0.5 M NaOH in 1.5 M NaCl for 15 min and neutralized in 0.5 M Tris–HCl, 1.5 M NaCl, pH 7 for 15 min. Gels were prehybridized in 50 ml of Church and Gilbert hybridization solution at 37 °C for 1 h. An aliquot (0.25 μg) of single-strand telomeric oligonucleotide $(TTAGGG)_3$ was end labeled with 50 μCi of γ^{32}P-ATP and 10 U of T4 polynucleotide kinase and added to the prehybridization buffer. The gels were incubated at 37 °C for 16 h and washed 3 times with 0.5 × SSC, 0.1% SDS at 37 °C for 10 min each time. A Phospho-Imager was used to measure the position and strength of radioactive signal to determine TRF length. For a specific experiment, individual samples were run a single time in order that all pertinent data points could be compared on a single gel, and comparisons were made only from data run on the same gel. If there was enough sample to run a duplicate gel for comparison, the difference in average telomere length was less than 0.5 kb for identical samples.

Cell synchronization and cell cycle analysis

Neurosphere cultures were treated with 5 μg/ml aphidicolin (Sigma, St. Louis, MO) for 24 h, washed 3 times with basal media with the appropriate combination of growth factors and cultured for an additional 24 h in that same media. In preparation for cell cycle analysis, treated neurospheres or control unsynchronized cultures were incubated with 0.1% trypsin–EDTA for 10 min at 37 °C to make a single cell suspension, fixed in 70% ethanol, washed with PBS, incubated with 20 μg/ml RNase and 5 μg/ml propidium iodide in PBS containing 0.1% bovine serum albumin and 0.5% Tween 20. Cell cycle analysis was performed using a FACSCaliber flow cytometer (Becton Dickson, San Jose, CA).

Preparation of cell-free lysates and immunoblotting

Neurospheres were isolated, suspended in 300 μl of lysis buffer [58], incubated on ice for 15 min and centrifuged at 13,000 rpm for 15 min at 4 °C. Protein concentration was determined using DC protein assay (Biorad, Richmond, VA). Protein from cell-free lysates (40 μg) was separated on a 15% SDS-polyacrylamide gel, electro-transferred to PDVF membrane, blocked with 5% dry milk in Tris-buffered saline containing 0.01% Tween 20, probed with primary antibodies toward p21 (polyclonal, 1:500, Santa

Cruz Biotechnology, Santa Cruz, CA) and actin (monoclonal, 1:500, Sigma, St. Louis, MO) and then incubated with a secondary antibody conjugated to horseradish peroxidase (Promega. Madison, WI). Antibody labeling was visualized using a chemiluminescence kit (Amersham, Piscataway, NJ).

Population doubling time analysis

To determine the number of cells per neurosphere, individual neurospheres (n = 30) were transferred to wells in a 96-well plate and the diameters measured with a lens-mounted micrometer. Prewarmed Accutase (100 μl) was added to each well, and cells were incubated for 10 min at 37 °C, triturated and counted using trypan blue. Neurosphere volume calculated from the diameter measurements was plotted against total number of cells, and the resulting standard curve was used to calculate the number of cells (y) per neurosphere volume (x). This has previously been shown to provide an accurate estimate of cell number per sphere [25]. Data generated during the growth curve experiment were analyzed to determine population doubling time (PD). Diameter measurements taken at days 6 and 10 for lines E, F and G were used to estimate cell number per sphere. PD was calculated using the formula, $N = N_0 2^{T/\mathrm{mgt}}$, where N is the final cell number, N_0 is the initial cell number, T is the elapsed time, and mgt is the mean generation time [59].

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2006.03.012.

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